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(54) Title: SPONGIFORM ENCEPHALOPATHY DETECTION METHODS

(57) Abstract

A method for detecting the presence of spongiform encephalopathy in an animal comprising determining the presence and/or amount of agent (e.g. by the use of 2DPAGE, followed by staining and densitometry readings of the stained agent) in a body fluid (e.g. cerebrospinal fluid) of test animal which cross-reacts with antibody raised against apoliprotein E, and has a molecular weight of between 34 and 38 kDa and a pI of between 5.4 and 5.7 comparing the concentration with a control value, and correlating the relationship between the two with the likely presence of spongiform encephalopathy in the animal.

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SPONGIFORM ENCEPHALOPATHY DETECTION METHODS

The present invention relates to methods for the detection of spongiform encephalopathies in animals, and in particular for the detection of bovine spongiform encephalopathy (BSE) in cattle.

Spongiform encephalopathies are a group of diseases which include scrapie in sheep and Creutzfeldt-Jakob disease (CJD) in humans.

BSE is a notifiable fatal neurodegenerative disease found in cattle. BSE is of major importance to the British farming industry.

Currently cases of BSE are identified by clinical manifestations in the animal. Cases are confirmed by post-mortem analysis of brain tissue, for instance by histopathology, by detection of scrapie associated fibrils or proteinase K resistant protein.

These methods have the disadvantage that they necessitate the slaughter of potentially-infected animals which may turn out to be disease-free. Alternatively, clinical signs may be absent or go undetected, thus leaving infected animals in the herd.

Harrington et al (New England Journal of Medicine (1986) Vol 315, No 5, pp 279-283) used high resolution two dimensional polyacrylamide gel electrophoresis (2DPAGE) to discover the presence of 4 abnormal proteins in the cerobrospinal fluid of human patients suffering from CJD. However, the precise identity of these proteins was not ascertained.

Thus there exists a need for a pre-mortem test for spongiform encephalopathies which can be used when diagnosing potentially-infected animals.

The present invention has now provided a method for detecting spongiform encephalopathies in animals which addresses some, and in preferred forms all, of these problems.

According to one aspect of the present invention there is provided a method for detecting the presence of spongiform encephalopathy in an animal comprising determining the presence and/or amount of agent in a body fluid of the animal which cross-reacts with antibody raised against apoliprotein E, and relating the result of this determination to the infection status of the animal.

Preferably the result of the determination is compared with a control value, and the relationship between the two is correlated with the infection status of the animal.

Preferably the method is used to detect BSE.

Apolipoprotein E is a cholesterol transporting protein produced in the peripheral and central nervous system. Its presence in either multiple- or single-forms has been categorised in cerebrospinal fluid (CSF) and serum.

Thus the discovery that spongiform encephalopathy infection in an animal can be correlated with the presence of, or an increase in the concentration of, an agent or agents in the body fluids of that animal, forms the basis for the methods of the current invention.

The agent or agents are cross reactive with anti-apolipoprotein E, have a molecular weight of around 34-38 kDa, and have a pI of around 5.4 - 5.7. This is consistent with their identification as apolipoprotein E, and the term 'Apo E agent' as used hereinafter is intended to embrace any agent which has these properties (including apolipoprotein E itself and isoforms or multiple-forms thereof).

It should be noted that there is no requirement to accurately quantify the Apo E agent concentration because spongiform encephalopathy may be detected by comparison with a control.

The control value may be derived from the Apo E agent concentration in a different animal (for which the infection status is known) and which is analysed in parallel with the test animal. Alternatively,

the control value may come from the same animal, or be a known standard.

The control value may be determined using the same method used for the test animal, or may be derived by a different analytical method.

The results from the 'control' animal may be used to derive a standard positive- or negative-control value, or to calibrate the test animal result.

Preferably the body fluid analysed in the method is CSF since authentic apolipoprotein E is the major apolipoprotein found in this fluid. Additionally, the proximity of the CSF to brain means that neurological disorders which produce alterations in the protein composition of the brain may be manifested in the CSF. Methods for extracting samples of CSF are well known to those skilled in the art.

The invention embraces any method for analysing the concentration of Apo E agent in a body fluid of an animal which is currently comprised in the art, and any methods which may later become available.

Preferably the presence and/or amount of Apo E agent in a body fluid of the animal is derived by the use of PAGE or 2DPAGE to separate out the Apo E agent from other agents in the body fluid, and then staining the gel and making densitometry measurements in the region of the gel of interest in order to determine the presence and/or amount of the Apo E agent.

Preferably the identity of the Apo E agent is confirmed by use of immunogenic material, for instance antibody raised against Apo E agent or a synthetic peptide based on a sequence thereof. Suitable immunogen-based techniques for identifying the presence of cross-reactive agents are well known to those skilled in the art eg. ELISA or Western Blotting.

In alternative embodiments of the invention, these immunogenic techniques may be used both to identify the Apo E agent, and

also to estimate its concentration.

Thus the invention makes available methods for detecting the presence of spongiform encephalopathy in a test animal which address many, and in preferred forms all, of the problems of the prior art. The balance between test certainty and ease of use will be dependent on the precise method of Apo E agent analysis chosen for use in the methods of the current invention. However, the pre-mortem diagnosis of BSE in cattle opens up the possibility of mass-testing in herds, thereby reducing the likelihood of slaughtering uninfected animals or 'missing' infected ones.

The methods of the present invention will now be described, by way of illustration only, through reference to the following example and figures. Other embodiments falling within the scope of the invention will occur to those skilled in the art in the light of this.

EXAMPLE - IDENTIFICATION OF BSE IN CATTLE

Sample preparation: CSF samples were collected from BSE-positive cattle and BSE-negative cattle. In each case the diagnosis was confirmed by post-mortem histopathology and electron microscopy. CSF samples were taken by cisternamagna puncture after death and concentrated 10-15 fold. Volumes of CSF containing 40 µg total protein were mixed in a 4:1 ratio with denaturing solution (1g sodium dodecyl sulphate (SDS) and 0.232g dithiothreitol in 10ml water) and heated at 95°C for 5 minutes. Samples were then pulse centrifuged.

Electrophoresis: The prepared samples were 2D electrophoresed using a Millipore Investigator 2D electrophoresis system according to the method in the instruction manual. First dimensional iso-electric-focussing was carried out in 26 cm threaded glass tubes with 1 mm inner diameter in a pH gradient of 3-10 for 18000 volt hours after pre-focussing the gels for 1 hour to 1500 V. Second dimension SDS-PAGE was carried out using 1 mm thick large format gels (23 cm x 23 cm) with 12.5% acrylamide and no stacking gel.

Staining and Image analysis: The 2D gels were silver stained according to the Millipore manual. Gels were scanned with an Omnimedia scanner XRS and analysed using Bioimage software and Investigator Database programme (Millipore) using a sunSPARC station computer.

Confirmation of the identity of Apo E agent: The 2D gels were electroblotted onto Immobilon-P membranes overnight at 30V using a Bio-Rad Trans-Blot cell. The blots were blocked using Tween 80 for 1 hour and then incubated for 90 minutes with sheep antiserum containing polyclonal antibody raised against authentic apolipoprotein E. Bound sheep antibodies were detected using rabbit anti-sheep IgG and a horseradish peroxidase detection system. A number of agents in the region of interest (approximate molecular weight of 34-38 kDa, and a pI of around 5.4 - 5.7) were found to have cross reacted with anti-apolipoprotein E antibody.

Comparison of BSE-negative and BSE-positive cattle: A comparison of the stained gels from typical BSE-positive and -negative samples is shown in Fig 1(a) and Fig 1(b). As can be seen the number and intensity of the silver stained spots in the region corresponding to agents having an approximate molecular weight of 34-38 kDa, and a pI of around 5.4 - 5.7 (labelled 'Apo E') is higher in the BSE-positive sample.

A comparison of the mean optical density of those silver-stained spots on the gels which were also found to cross react with anti-apolipoprotein E antibody is found below:

Agent No	BSE-negative	BSE-positive
1	0.13	0.47
2	0.42	0.84
3	0.45	0.64
4	0.45	0.99
5	0.46	1.02
6	0.41	0.69
7	0.87	1.12

By comparing the two sets of optical density readings it can be seen that each agent is present in consistently higher amounts in the BSE-positive (n=31) animals than in the BSE-negative (n=27) animals, thus indicating that the presence and/or amount of these agents can be used to detect the likely presence of BSE in potentially infected animals.

CLAIMS

1. A method for detecting the presence of spongiform encephalopathy in an animal comprising determining the presence and/or amount of agent in a body fluid of the animal which cross-reacts with antibody raised against apoliprotein E, and relating the result of this determination to the infection status of the animal.

2. A method as claimed in claim 1 wherein the result of the determination is compared with a control value, and the relationship between the two is correlated with the infection status of the animal.

3. A method as claimed in claim 1 or claim 2 wherein the agent has a molecular weight of between 34 and 38 kDa and a pI of between 5.4 and 5.7

4. A method as claimed in any one of the preceding claims wherein the agent is apolipoprotein E.

5. A method as claimed in any one of the preceding claims wherein the spongiform encephalopathy is bovine spongiform encephalopathy.

6. A method as claimed in any one of the preceding claims wherein the body fluid analysed in the method is cerebrospinal fluid.

7. A method as claimed in any one of the preceding claims wherein the presence and/or amount of agent in a body fluid of the animal is determined by substantially separating the agent from other materials in the body fluid of the animal using polyacrylamide gel electrophoresis, staining the gel, identifying the agent, and determining the presence and/or amount of the agent from the density of the staining of the agent.

8. A method as claimed in claim 7 wherein the polyacrylamide gel

electrophoresis is two-dimensional polyacrylamide gel electrophoresis.

9. A method as claimed in any one of the preceding claims wherein the identity of the agent is confirmed by use of immunogenic material.

10. A method as claimed in any one of claims 1 to 6 wherein the presence and/or amount of agent in a body fluid of the animal is determined by by the use of immunogenic material.

11. A method as claimed in claim 9 or 10 wherein the immunogenic material is antibody raised against apolipoprotein E.

12. A method as claimed in any one of claims 2 to 11 wherein the control value is derived by the same method as that used with the animal but using a further animal known to be either spongiform encephalopathy-negative or -positive.

13. A method for detecting the presence of spongiform encephalopathy in an animal substantially as described hereinbefore.

Fig.1(b).

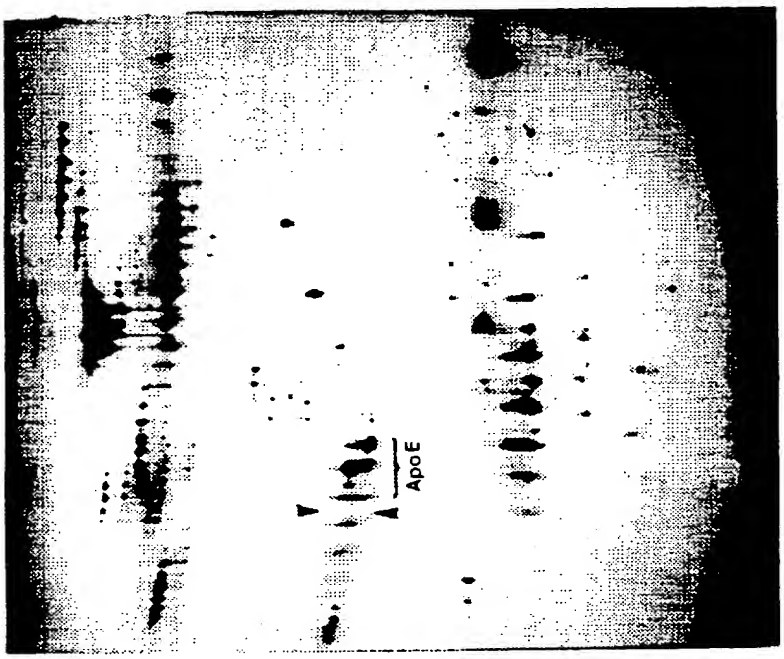
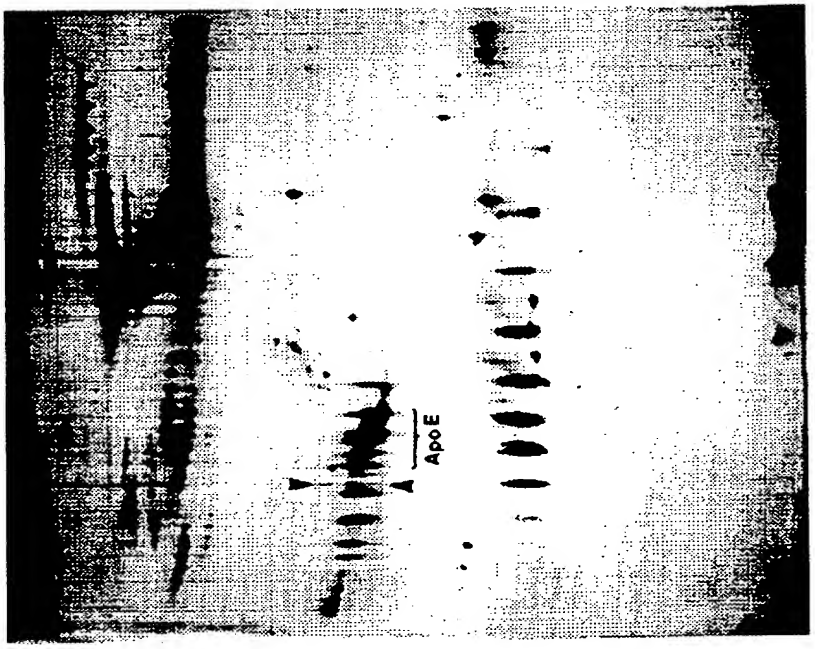


Fig.1(a).



INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/68 G01N33/569

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHEMICAL ABSTRACTS, vol. 120, no. 25, 20 June 1994 Columbus, Ohio, US; abstract no. 320660v, page 634; column 2; see abstract & SHINKEI KENKYU NO SHINPO, vol. 37, no. 6, - 1993 TOKYO, pages 1039-1051, Y. NAMBA 'Immunochemical demonstration of apolipoprotein E in cerebral amyloid deposits in Alzheimer's disease and kuru plaque amyloids in Creutzfeldt-Jacob disease.' see the whole document --- -/--	1-13

[X] Further documents are listed in the continuation of box C.

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Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US,A,4 892 814 (M.G. HARRINGTON ET AL.) 9 January 1990 see the whole document	1-13
A	JOURNAL OF VIROLOGY, vol. 65, no. 9, 1 September 1991 WASHINGTON DC USA, pages 4759-4768, XP 000567293 J.F. DIEDRICH ET AL. 'Neuropathological changes in scrapie and Alzheimer's disease are associated with increased expression of Apolipoprotein E and cathepsin in astrocytes.' see page 4764, column 1, line 16 - line 38; figure 4	1-13
A	NEW ENGLAND JOURNAL OF MEDICINE, vol. 315, no. 2, 31 July 1986 BOSTON MA USA, pages 279-283, XP 000567332 M.G. HARRINGTON ET AL. 'Abnormal proteins in the cerebrospinal fluid of patients with Creutzfeldt-Jacob disease.' cited in the application see the whole document	1-13

INTERNATIONAL SEARCH REPORT

.formation on patent family members

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-4892814	09-01-90	NONE	